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(54) Title: METHODS FOR INHIBITING CELL PROLIFERATION BY INHIBITING THE MITOGENIC ACTIVITY OF MACROPHAGE MIGRATION INHIBITORY FACTOR

(57) Abstract

This invention is directed to methods of inhibiting cell proliferation by inhibiting the mitogenic activity of macrophage migration inhibitory factor ("MIF"). Inhibitory nucleic acids directed against MIF inhibit cell growth in both cells induced by growth factors and transformed (malignant) cells that express oncogenes.

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METHODS FOR INHIBITING CELL PROLIFERATION BY INHIBITING THE MITOGENIC ACTIVITY OF MACROPHAGE MIGRATION INHIBITORY FACTOR

This application is a continuation-in-part of U.S. Serial No. 08/340,826, filed November 16, 1995, incorporated herein by reference.

BACKGROUND OF THE INVENTION

This invention relates in general to the fields of cellular and molecular biology and, in particular, to methods of inhibiting the growth of cells by inhibiting the mitogenic activity of macrophage migration inhibitory factor.

The control of cell growth is of interest to scientists in the understanding of normal physiological activity, such as erythropoiesis and wound healing, and of pathological conditions, such as cancer. In this regard, the relationship between peptide growth factors and oncogenes, genes whose expression causes cells to become cancerous, has caught the attention of scientists. (See, e.g., M. Sporn et al., Principals of Cancer Biology, Chapter 3 in <u>Cancer -- Principals & Practice of Oncology, 2nd Ed.</u>, V.T. DeVita, S. Hellman and S.A. Rosenberg, Eds., J.B. Lippencott Company, Philadelphia (1985).) Certain mechanisms of cell proliferation in cancer appear to mimic the growth factor-induced mitogenic pathway. Researchers have found, for example, that certain oncogenes, such as *src*, encode a protein kinase activity that resembles the protein kinase activity induced by certain growth factors. Studies have also shown that some peptide growth factors, such as PDGF, induce the expression of oncogenes; and certain malignant cells require less stimulation by growth factors than normal cells to proliferate.

Peptide growth factors act by binding to receptors on the cell surface. From there, the induction of mitogenesis

involves a complex and only partially understood sequence of intra-cellular signals and events. Growth factors induce the expression of many genes, some of which are activated within minutes, called primary response genes, and others which are activated after a few hours, called delayed early response genes. (See, e.g., Lanahan et al., (1992) *Molec. and Cell Biol.*, 12:3919-3929.) The function of many of the proteins encoded by these genes is unknown.

Among the many genes induced by these growth factors is one encoding macrophage migration inhibitory factor ("MIF"). MIF was originally identified as a lymphokine secreted by T cells at a site of infection. Upon contact with monocytes, MIF causes them to stick to the blood vessel endothelium where the cells force themselves through the vessel lining into the surrounding tissue. There, monocytes may differentiate into macrophages. Since the discovery that T cells produce MIF, it was discovered that the protein is synthesized by non-immune cells, for example, in the developing eye lens, where its function is unknown. (G. Wistow et al., (1993) *Proc. Natl. Acad. Sci., USA*, 90:1272-1275.

There exists a need in the art for methods of intervening in the mitogenic pathway induced by growth factors and already active in cancer cells.

SUMMARY OF THE INVENTION

This invention satisfies this need and provides related advantages as well by demonstrating that one of the genes induced by growth factors, macrophage migration inhibitory factor, or "MIF," is involved in cell proliferation and that inhibition of MIF expression inhibits both peptide growth factor-induced and transformed cell proliferation.

This invention provides methods useful for inhibiting the growth of a cell involving the step of inhibiting the mitogenic activity of MIF in the cell. The methods can be used both *in vitro* to control growth of transformed cells or cells stimulated growth factors, or the method can be used *in vivo* in therapeutic methods.

According to one embodiment of the invention, the step of inhibiting the mitogenic activity of MIF involves providing the cell with an inhibitory nucleic acid that inhibits expression of MIF. The inhibitory nucleic acid can be a sense or an antisense nucleic acid that inhibits transcription of the MIF gene or translation of MIF mRNA. Antisense nucleic acids can be directed, for example, against a target sequence in the sense strand of the MIF gene, a target sequence spanning the boundary between an intron and an exon of MIF pre-mRNA, or, in a preferred embodiment, a target sequence in MIF mRNA. The antisense nucleic acid also can be a ribozyme that cleaves MIF mRNA. This invention also provides isolated forms of these inhibitory nucleic acids.

This invention contemplates delivering inhibitory nucleic acids to the cell by introducing an expression vector having an expression control sequence operatively linked to a nucleic acid sequence encoding the inhibitory nucleic acid. It also contemplates contacting the cell with the inhibitory nucleic acid, for example, by administration to the external milieu of the cell.

According to another embodiment of the invention, the step of inhibiting MIF activity involves inhibiting the binding of MIF to retinoblastoma ("Rb") protein.

This invention is also directed to therapeutic methods useful for inhibiting the growth of a malignant cell in an individual involving administering to the individual a therapeutically effective amount of an agent that inhibits the mitogenic activity of MIF in the malignant cell. The agent can be an inhibitory nucleic acid or a compound that interferes with MIF binding to Rb protein. In a preferred embodiment, the agent is injected into a tumor mass. This invention also provides pharmaceutical compositions having an agent that inhibits the mitogenic activity of MIF and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents results of PAGE on cell extracts from NIH 3T3 cells grown in the presence and absence of PDGF

and TGFβ1. Treatment of serum-free NIH 3T3 cells with either PDGF or TGFβ1 results in stimulation of MIF expression.

Fig. 2 shows stimulation of the human MIF gene promoter by PDGF and TGFβ1. NIH 3T3 cells were stably transfected with plasmid containing the human MIF gene promoter (V. Paralkar and G. Wistow, (1994) *Genomics* 19:48-51) cloned upstream of the bacterial reporter gene chloramphenicol acetyltransferase (CAT). Treatment of these cells resulted in four-fold induction of CAT activity over serum-free control in the presence of serum and 2-3 fold in the presence of TGFβ1 and PDGF, respectively. Similar results were seen in transient transfection experiments. CAT activity relative to serum-free control is shown as mean values + standard deviation for three individual measurements.

Figs. 3A-3B show the effect of treatment with antisense MIF oligonucleotide in abolishing cell proliferation and synthesis of MIF protein.

Fig. 3A shows ^{3}H-thymidine incorporation in NIH 3T3 cells treated with growth factors and antisense or control oligonucleotides. Cells were grown in the presence of 0.2% serum and then exposed to (a) no additional treatment, (b) PDGF (5 ng/ml), (c) PDGF + aMIF1 (10 μM), (d) PDGF + sMIF1 (10 μM), (e) PDGF + aMIF2 (10 μM), (f) PDGF + 1FIMa, (g) TGFβ1 (10 ng/ml) and (h) TGFβ1 + aMIF1. Antisense treated cells recovered, i.e., they regained the ability to grow after washing with fresh medium: (i) control cells were grown for 48 hr in 10% serum, (j) cells were first treated with PDGF + aMIF1 for 24 hours as in part (c) then medium was replaced with 10% serum for a further 24 hours. ^{3}H-thymidine incorporation is shown relative to value for lane (a). Each point represents mean ± standard deviation for three measurements. The assay was repeated with multiple preparations of oligos synthesized on two different Applied Biosystems synthesizers giving similar results.

Fig. 3B shows a western blot of MIF protein in NIH 3T3 cells grown in the presence of PDGF and TGFβ1 and presence or absence of aMIF1 using antisera to human MIF. Serum-free NIH 3T3 cells were cultured in the presence of PDGF

(10 ng/ml) or TGFβ1 (15 ng/ml) and presence or absence of aMIF1 (10 μM) overnight. Cells were lysed at 4°C in a buffer containing 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM PMSF, 20 μg/ml aproteinin, 20 μg/ml leupeptin. Cell lysates were clarified by centrifugation and their protein concentration determined. Protein extracts (10 μg/treatment) were subjected to SDS PAGE (Novex, San Diego, CA), transferred to nitrocellulose filters and analyzed by western blotting with chicken anti-human MIF antiserum. This antiserum was raised as an IgY fraction in chickens immunized with a fusion protein of human MIF and maltose binding protein. Visualization was by enhanced chemiluminescence (Amersham, Arlington Heights, IL), using goat anti-chicken immunoglobulin secondary antibody (Vector labs).

Figs. 4A-4B demonstrate that MIF acts near the G1/S boundary and interacts with Rb protein.

Fig. 4A shows a western blot of cyclins in NIH 3T3 cells grown in the presence of PDGF and presence or absence of aMIF1 using anti-cyclin antibodies. Non-specific bands are Ig bands from immunoprecipitation.

Fig. 4B shows a flow cytometry analysis of aMIF1 treated cells: a) control NIH 3T3 cells in the absence of serum; b) serum-free cells grown in the presence of 5 ng/ml PDGF; c) PDGF-treated cells in the presence of aMIF1. Percentages of cells in G1, S and G2/M phases of the cell cycle are indicated. Cells were grown as described herein. Flow cytometry was performed as described in V.V. Ogryzko et al. (1994) *J. Virol.* 68:3724, using a FACscan flow cytometer equipped with a doublet discrimination (Becton-Dickinson).

Fig. 5 demonstrates that constitutive expression of mouse MIF shortens G1 and allows cell proliferation independent of serum. It shows results of: a) flow cytometry of NIH 3T3 cells stably transformed with recombinant mouse MIF cDNA grown in 10% serum; (b) control cells in serum; c) MIF-constitutive cells in serum-free medium; (d) control cells in serum-free medium.

Fig. 6A shows a western blot of MIF in immunoprecipitates of NIH 3T3 cell lysates using antisera to

cell cycle components which control G1/S transition. Rabbit immunoglobulin-derived bands from the immunoprecipitation were detected in all lanes. Lane C shows results for western blot of anti-Rb antibody in the absence of cell lysate as control. Lane 1 shows western blot of proteins immunoprecipitated with agarose conjugated antiserum to Rb, retinoblastoma protein; Lane 2 shows western blot of protein immunoprecipitated with anti-cyclin E in the presence of agarose conjugated protein A; Lane 3 shows western blot of proteins immunoprecipitated with agarose conjugated anti-CDK2. All lanes were subjected to western analysis with chicken anti-human MIF.

Fig. 6B shows a western blot of MIF in proteins immunoprecipitated by agarose conjugated anti-Rb under non-reducing conditions. As in part (a), lane C is control without cell lysate while lane 1 contains anti-Rb immunoprecipitate. The immunoglobulin-derived bands now migrate at larger size appropriate for intact antibodies. MIF reactive protein in the anti-Rb immunoprecipitate migrates at a size appropriate for a homodimer under non-reducing conditions.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

This invention is directed to new methods of inhibiting mitogenesis in a cell. The invention results from the discovery that MIF has hitherto unrecognized mitogenic functions and that the inhibition of MIF activity in a cell results in the inhibition of cell proliferation. In particular, mitogenesis normally induced by stimulation with growth factors, such as Platelet-Derived Growth Factors (PDGF) and Transforming Growth Factors-ß (TGF-ß), and mitogenesis in cells transformed with the oncogenes *ras*, *raf* and *sis*, are inhibited when MIF activity is inhibited with antisense polynucleotides.

PDGF and TGF-ß1 act through different signaling pathways: tyrosine kinase receptors and serine/threonine kinase receptors, respectively. Oncogenes *ras*, *raf* and *sis* act as components early in the signalling pathways that lead to cell division. The fact that inhibition of MIF halts cell

division in all these pathways indicates that MIF acts late in the cell cycle after these pathways converge.

Results described herein show that MIF binds to the retinoblastoma ("Rb") protein in the cell and is required for entry of the cell into the S phase of the cell cycle. (See, e.g., Wiman, (1993) *FASEB J.*, 10:841-845 and Cobrinik et al., (1992) *Trends Biochem. Res.*, 17:312-315.) Inhibiting MIF activity arrests cells in G1 phase, and they cease proliferation.

Accordingly, this invention provides methods that result in the inhibition of cell growth involving inhibiting the mitogenic activity of MIF in a cell, both *in vitro* and *in vivo*. The inhibition of MIF activity of cells *in vitro* is useful as a research tool to investigate the complex sequence of events that regulate the cell cycle. The inhibition of MIF activity in cells *in vivo* is useful in therapeutic methods for inhibiting the growth of malignant cells.

In vitro, the methods of this invention are applicable to any culture cells whose growth has been stimulated by growth factors, such as, for example, PDGF, TGF-β, Fibroblast Growth Factor ("FGF") or the Insulin-like Growth Factors ("IGF"). It is also applicable to cells from transformed cell lines, including cells transformed by the introduction of oncogenes.

In vivo, the methods of this invention are applicable, for example, to malignant cells. In particular, this invention contemplates inhibiting the growth of cells whose malignancy involves uncontrolled progression through S phase, including those cells expressing oncogenes such as *c-ras*, *c-raf* and *c-sis*. The methods of this invention are also applicable to inhibiting the growth of cells *in vivo* whose growth is induced by growth factors.

As used herein, "MIF" or "macrophage migration inhibitory factor" means the human protein having lymphokine activity and having the DNA sequence [SEQ ID NO:1] and deduced amino acid sequence [SEQ ID NO:2] presented in Table I, its alleles and any mammalian cognates of it. The sequence in Table I is from V. Paralkar and G. Wistow, (1994) *Genomics*,

19:48-51 (GenBank Accession No. L19696). "#" indicates the transcription start site. "*" indicates the beginning and end of introns. "@" indicates the polyadenylation site (AATAAA). The sequence of other MIF cDNAs are also known. (See, e.g., the following MIF sequences in GenBank: HSMMIHFA; HUMLMIF; HUMMIFA; MMMMIHFA; MUS10KMIF; CHKLMIF; HUMGIA; and MUSGIA. The last two are identified as glycosylation-inhibiting factor.)

TABLE I

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1    CTGCAGGAAC CAATACCCAT AGGCTATTTG TATAAATGGG CCATGGGGCC TCCCAGCTGG
61   AGGCTGGCTG GTGCCACGAG GGTCCCACAG GCATGGGTGT CCTTCCTATA TCACATGGCC
121  TTCACTGAGA CTGGTATATG GATTGCACCT ATCAGAGACC AAGGACAGGA CCTCCCTGGA
181  AATCTCTGAG GACCTGGCCT GTGATCCAGT TGCTGCCTTG TCCTCTTCCT GCTATGTCAT
241  GGCTTATCTT CTTTCACCCA TTCATTCATT CATTCATTCA TTCAGCAGTA TTAGTCAATG
301  TCTCTTGATA TGCCTGGCAC CTGCTAGATG GTCCCCGAGT TTACCATTAG TGGAAAAGAC
361  ATTTAAGAAA TTCACCAAGG GCTCTATGAG AGGCCATACA CGGTGGACCT GACTAGGGTG
421  TGGCTTCCCT GAGGAGCTGA AGTTGCCCAG AGGCCCAGAG AAGGGGAGCT GAGCACGTTT
481  GAACCACTGA ACCTGCTCTG GACCTCGCCT CCTTCCTTCG GTGCCTCCCA GCATCCTATC
541  CTCTTTAAAG AGCAGGGGTT CAGGGAAGTT CCCTGGATGG TGATTCGCAG GGGCAGCTCC
601  CCTCTCACCT GCCGCATGAC TACCCCGCCC CATCTCAAAC ACACAAGCTC ACGCATGCGG
661  GACTGGAGCC CTTGAGGACA TGTGGCCCAA AGACAGGAGG TACAGGGGCT CAGTGCGTGC
721  AGTGGAATGA ACTGGGCTTC ATCTCTGGAA GGGTAAGGGG CCATCTTCCG GGTTCACCGC
781  CGCATCCCCA CCCCCGGCAC AGCGCCTCCT GGCGACTAAC ATCGGTGACT TAGTGAAAGG
841  ACTAAGAAAG ACCCGAGGCG AGGCCGGAAC AGGCCGATTT CTAGCCGCCA AGTGGAGAAC
901  AGGTTGGAGC GGTGCGCCGG GCTTAGCGGC GGTTGCTGGA GGAACGGGCG GAGTCGCCCA
961  GGGTCCTGCC CTGCGGGGGT CGAGCCGAGG CAGGCGGTGA CTTCCCCACT CGGGGCGGAG

                                                             #
1021 CCGCAGCCTC GCGGGGGCGG GGCCTGGCGC CGGCGGTGGC GTCACAAAAG GCGGGACCAC

1081 AGTGGTGTCC GAGAAGTCAG GCACGTAGCT CAGCGGCGGC CGCGGCGCGT GCGTCTGTGC

                                  M  P  M   F  I  V   N  T  N
1141 CTCTGCGCGG GTCTCCTGGT CCTTCTGCCA TCATGCCGAT GTTCATCGTA AACACCAACG

      V  P  R  A   S  V  P   D  G  F   L  S  E  L   T  Q  Q   L  A  Q
1201 TGCCCCGCGC CTCCGTGCCG GACGGGTTCC TCTCCGAGCT CACCCAGCAG CTGGCGCAGG

      A  T  G  K   P  P  Q   *
1261 CCACCGGCAA GCCCCCCCAG GTTTGCCGGG AGGGGACAGG AAGAGGGGGG TGCCCACCGG

1321 ACGAGGGGTT CCGCGCTGGG AGCTGGGGAG GCGACTCCTG AACGGAGCTG GGGGGCGGGG

1381 CGGGGGGAGG ACGGTGGCTC GGGCCCGAAG TGGACGTTCG GGGCCCGACG AGGTCGCTGG

                                * Y  I  A  V   H  V  V   P  D  Q
1441 GGCGGGCTGA CCGCGCCCTT TCCTCGCAGT ACATCGCGGT GCACGTGGTC CCGGACCAGC

      L  M  A  F   G  G  S   S  E  P   C  A  L  C   S  L  H   S  I  G
1501 TCATGGCCTT CGGCGGCTCC AGCGAGCCGT GCGCGCTCTG CAGCCTGCAC AGCATCGGCA

      K  I  G  G   A  Q  N   R  S  Y   S  K  L  L   C  G  L   L  A  E
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1561 AGATCGGCGG CGCGCAGAAC CGCTCCTACA GCAAGCTGCT GTGCGGCCTG CTGGCCGAGC

      R  L  R  I   S  P  D   R*
1621 GCCTGCGCAT CAGCCCGGAC AGGTACGCGG AGTCGCGGAG GGGCGGGGGA GGGGCGGCGG

                                                              * V
1681 CGCGCGGCCA GGCCCGGGAC TGAGCCACCC GCTGAGTCCG GCCTCCTCCC CCCGCAGGGT

        Y  I  N   Y  Y  D   M  N  A  A   N  V  G   W  N  N   S  T  F  A
1741 CTACATCAAC TATTACGACA TGAACGCGGC CAATGTGGGC TGGAACAACT CCACCTTCGC

1801 CTAAGAGCCG CAGGGACCCA CGCTGTCTGC GCTGGCTCCA CCCGGGAACC CGCCGCACGC

                                                 @
1861 TGTGTTCTAG GCCCGCCCAC CCCAACCTTC TGGTGGGGAG AAATAAACGG TTTAGAGACT

1921 AGGAGTGCCT CGGGGTTCCT TGGCTTGCGG GAGGAATTGG TGCAGAGCCG GGACATTGGG

1981 GAGCGAGGTC GGGAAACGGT GTTGGGGGCG GGGGTCAGGG CCGGGTTGCT CTCCTCGAAC

2041 CTGCTGTTCG GGAGCCCTTT TGTCCAGCCT GTCCCTCCTA CGCTCCTAAC AGAGGAGCCC

2101 CAGTGTCTTT CCATTCTATG GCGTACGAAG GGATGAGGAG AAGTTGGCAC TCTGCCCTGG

2161 GCTGCAG
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This invention contemplates inhibiting MIF activity in a cell by, for example, inhibiting MIF expression with inhibitory nucleic acids or by inhibiting the binding of MIF to Rb protein.

According to certain embodiments of this invention methods for inhibiting the mitogenic activity of MIF in a cell involve providing the cell with an inhibitory nucleic acid that inhibits expression of MIF. Inhibitory nucleic acids are single-stranded nucleic acids which can specifically hybridize to a target nucleic acid sequence. Typically, hybridization will occur when there is at least about 55% complementarity over a stretch of at least 14-25 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See M. Kanehisa, *(1984) Nucleic Acids Res.* 12:203.

By binding to the appropriate target sequence, an RNA-RNA, DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene. Recently, approaches for use of "sense" inhibitory nucleic acids have also been developed. The term "inhibitory nucleic acids" as used herein, refers to both "sense" and "antisense" nucleic acids.

By binding to the target nucleic acid, the inhibitory nucleic acid can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking DNA replication or transcription; interfering with processing of, poly(A) addition to, or translation of mRNA; or promoting inhibitory mechanisms of the cells, such as promoting RNA degradation. Inhibitory nucleic acid methods therefore encompass a number of different approaches to altering expression of specific genes that operate by different mechanisms. These different types of inhibitory nucleic acid technology are described in C. Helene and J. Toulme, (1990) *Biochim. Biophys. Acta.*, 1049:99-125, which is referred to hereinafter as "Helene and Toulme".

Inhibitory nucleic acid approaches can be classified into those that target DNA sequences, those that target RNA sequences (including pre-mRNA and mRNA), those that target proteins (sense strand approaches), and those that cause cleavage or chemical modification of the target nucleic acids.

Approaches targeting DNA fall into several categories. Nucleic acids can be designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. *See* Helene and Toulme.

Accordingly, this invention is directed to methods in which the inhibitory nucleic acid is directed against a target DNA sequence in the sense strand of the MIF gene. In particular, the target DNA sequence can span the site of initiation of transcription, nucleotide number 1077 of SEQ ID NO:1.

More commonly, inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are used to prevent maturation of pre-mRNA. Inhibitory nucleic acids can be designed to interfere with RNA processing, splicing or translation.

One approach is to target the inhibitory nucleic acids to U snRNP to thereby prevent the formation of

functional spliceosomes. Alternatively, inhibitory nucleic acids complementary to sequences at the boundary between introns and exons of the gene can be used to interfere with cleavage of pre-mRNA and ligation of exons.

Accordingly, this invention is directed to methods in which the inhibitory nucleic acid is directed against a sequence spanning the boundary between an intron and an exon of MIF pre-mRNA. Table I indicates the location of introns and exons in the MIF gene.

The inhibitory nucleic acids can also be targeted against mRNA. In this approach, the inhibitory nucleic acids are designed to hybridize to a target sequence in MIF mRNA and, thereby, interfere with its translation. Translation of mRNA is inhibited if there is a measurable decrease in the amount of MIF protein produced by the cell after treatment. Inhibiting translation of an mRNA results in suppression of the cellular function carried out by the encoded protein. For example, an inhibitory nucleic acid complementary to regions of c-myc mRNA inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which over-expresses the c-myc proto-oncogene. *See* E.L. Wickstrom et al., (1988) *PNAS (USA)*, 85:1028-1032 and Harel-Bellan et al., (1988) *Exp. Med.*, 168:2309-2318. Maier et al., (1990) *Science*, 249:1570-74 showed how to suppress interleukin-1α activity using unmodified antisense oligonucleotides. As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms in order to inhibit translation of the encoded protein(s).

In a preferred embodiment, this invention is directed to methods in which the inhibitory nucleic acid is directed against a target sequence in MIF mRNA. Inhibitory nucleic acids for inhibiting the translation of mRNA can have at least about 15 nucleotides. They also can be as long as the entire target mRNA. In one embodiment of the invention, the inhibitory nucleic acid is about 30 nucleotides long.

The activity of an inhibitory nucleic acid depends, in part, on the location of the target sequence in the mRNA. Inhibitory nucleic acids that hybridize to MIF mRNA at the

translation start site, i.e., that are directed against a sequence that includes the AUG start codon, are particularly effective in inhibiting translation of the MIF mRNA. In particular, this invention provides the antisense inhibitory nucleic acid having the sequence GATGAACATA GGCATGGTGG CGGAGAGACT [SEQ ID NO:3]. The underlined sequence, CAT, hybridizes to the target ATG start codon and, thereby, the polynucleotide spans the start codon. Other antisense inhibitory nucleic acids directed against the start codon include fragments of the aforementioned antisense nucleic acid of at least about 15 nucleotides, as well as other nucleic acids having a sequence that hybridizes to a sequence of about 15 nucleotides that span the start codon in the MIF mRNA sequence. As used herein, the verb "to have," when describing the sequence of a nucleic acid molecule, is used in the narrow sense to mean that the nucleic acid molecule has the sequence of nucleotides given without flanking sequences. Inhibitory nucleic acids directed against other parts of the MIF mRNA, particularly the 3' untranslated region, are less effective and, therefore, are to be avoided.

The inhibitory nucleic acids introduced into the cell can also encompass fragments of the "sense" strand of the gene or mRNA to trap or compete for the enzymes or binding proteins involved in mRNA translation. Of course, these fragments do not encode functional proteins or the target function would not be inhibited. See Helene and Toulme. Accordingly, this invention is directed to methods in which the inhibitory nucleic acid includes sequences from the sense strand of the MIF gene or MIF mRNA.

Also, this invention provides methods in which the inhibitory nucleic acids induces chemical inactivation or cleavage of the target genes or mRNA. Chemical inactivation can occur by the induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell. Alternatively, irreversible photochemical reactions can be induced in the target nucleic acid by means of a photoactive group attached to the inhibitory nucleic acid. Other chemical modifications of the target nucleic acids

induced by appropriately derivatized inhibitory nucleic acids can also be used. For example, the inhibitory nucleic acid can be attached to manganese or ferric ions. Thus, this invention is directed to methods wherein the inhibitory nucleic acid is attached to an agent that effects chemical inactivation of MIF mRNA.

Cleavage, and therefore inactivation, of the target nucleic acids can be effected by attaching a substituent to the inhibitory nucleic acid which can be activated to induce cleavage reactions. The substituent can be one that effects either chemical, photochemical or enzymatic cleavage.

Alternatively cleavage can be induced by the use of ribozymes or catalytic RNA. In this approach, the inhibitory nucleic acids would contain either naturally occurring RNA (ribozymes) or synthetic nucleic acids with catalytic activity. This invention also provides methods in which the inhibitory nucleic acid cleaves MIF mRNA. Bratty et al., (1992) *Biochim. Biophys. Acta.*, 1216:345-59 (1993) and Denhardt, (1992) *Ann. N.Y. Acad. Sci.*, 660:70-76 describe methods for making ribozymes.

One can synthesize inhibitory nucleic acids for use in the methods of this invention by any means known to the art including, for example, chemical synthesis, PCR and expression from expression vectors. Nucleic acid synthesis methods are well known to those of skill in the art. Inhibitory nucleic acids are chemically synthesized according to the solid phase phosphoramidite triester method first described by S.L. Beaucage and M.H. Caruthers, (1981) *Tetrahedron Letts.*, 22(20):1859-1862 using an automated synthesizer, as described in D.R. Needham-VanDevanter *et al.*, (1984) *Nucleic Acids Res.*, 12:6159-6168. Purification of nucleic acids is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in J.D. Pearson and F.E. Regnier, (1983) *J. Chrom.*, 255:137-149.

Inhibitory nucleic acids can also be produced by using polymerase chain reaction (PCR) technology. Appropriate primers and probes for amplifying the nucleic acid region of choice are generated from analysis of the DNA sequences.

Nucleic acid primers complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See *PCR Protocols: A Guide to Methods and Applications* (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990). The PCR amplified DNA can then be denatured and the antisense strand isolated by standard techniques known to those of skill in the art. See Sambrook, *et al.*, *supra*.

The sequence of the inhibitory nucleic acids can be verified using the chemical degradation method of Maxam and Gilbert, 1980, in Grossman, L. and Moldave, D., eds. Academic Press, New York, *Methods in Enzymology*, 65:499-560.

The inhibitory nucleic acids can be conventional nucleic acids, or are more commonly nucleic acids having properties which make them more desirable for inhibitory nucleic acid activity. For example, they can be made resistant to nucleases or more capable of specific binding to the desired target sequences. The specific binding can be effected by providing inhibitory nucleic acids having sequences which result in conventional base-pairing, or which recognize double-stranded DNA by binding to the major or minor grooves which are present in the DNA double helix. Alternatively, the inhibitory nucleic acids, in either single stranded or duplex form, can recognize target protein.

Whatever the ultimate strategy, it is desirable to provide oligomers with physiological properties which render them more effective. The general approach to constructing various nucleic acids useful in inhibitory nucleic acid therapy has been reviewed by A.R. Vander Krol *et al.* (1988), *Biotechniques* 6:958-976, and by C.A. Stein *et al.*, (1988) *Cancer Res.* (1988) 48:2659-2668. *See* also *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression,* Cohen, J.S., editor, MacMillan Press, London, pages 79-196 (1989), and Antisense RNA and DNA, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

There are a number of approaches described in the art whereby modified nucleic acids are used in these expanded antisense applications. A major impetus for modification of inhibitory nucleic acids is the nuclease susceptibility of unmodified single strand nucleic acids.

The phosphodiester linkages of native DNA and RNA molecules are readily degraded by exonucleases present in cells, tissue culture media, serum, blood and other body fluids. In order to provide enhanced stability *in vivo*, through resistance to endogenous nucleases, nucleic acids are synthesized with alternative linkages other than the conventional phosphodiester linkage. Among these are the methylphosphonates wherein one of the phosphorous-linked oxygens has been replaced by methyl phosphorothioates, wherein sulfur replaces one of the oxygens, and various amidates, wherein NH_2 or organic amino derivatives, such as morpholidates or piperazidates, replace an oxygen. Also carbonate and carbamate linkages can be employed, as well as those involving sulfur rather than oxygen as a linking substituent. Other modified internucleotide linkages can be synthesized that are generally nuclease resistant. *See* Miller *et al.*, (1981) *Biochemistry* 20:1874-1880, Letsinger *et al.*, (1986) *Nucleic Acids Res.* 14:3487-3499, Stein *et al.*, (1988) *Nucleic Acids Res.* 16:3209-3221, and Agrawal *et al.*, (1987) *Tet. Lett.* 28:3539-3542.

Nuclease stability can also be obtained through alternate modifications. For example, Haralambidis et *al.*, (1987) *Tet. Lett.* 28:5199-5202, describe the attachment of a peptide to the 3' hydroxyl terminus of a nucleic acid which was resistant to a 3' exonuclease (snake venom phosphodiesterase) under *in vitro* conditions.

Several additional modifications have been developed to render inhibitory nucleic acids resistant to exo- and endonucleases, including sulfur substitution of a deoxyribose phosphodiester oxygen (phosphorothioate nucleotides), and synthesis of the nucleosides in the (α)-anomeric configuration rather than the natural (β)-anomers. For example, the phosphorothioate modification of inhibitory nucleic acids is

used. This form, although resistant to most nucleases, does not interfere with RNase H cleavage of endogenous mRNA. RNase H degradation of the endogenous mRNA component of an (antisense) DNA:RNA hybrid can significantly amplify the inhibition of mRNA translation by the inhibitory nucleic acid caused by stearic hinderance.

The stability, nuclease resistance, and efficiency of the inhibitory nucleic acids can be improved by coupling an intercalating agent such as 2-methoxy, 6-chloro, 9-aminoacridine to the 3' or 5' end. The acridine conjugate can also promote the passage of the inhibitory nucleic acid out of the endocytic compartment and into the cytoplasm. Therefore, preferably, acridine-conjugated phosphothiorate antisense oligodeoxyribonucleotides with specificity for key activation and effector molecules in the targeted autoreactive T cells are constructed.

Intercalators and chelators which enhance the ability of the nucleic acid to bind the target DNA or RNA can also be introduced as a modification. These substituents can be attached to the 5' end of preconstructed nucleic acids using amidite or H-phosphonate chemistry, as described by K.K. Ogilvie *et al.* (1987) *Pure and Appl. Chem.*, 59:325, and by B.C. Froehler (1986) *Nucleic Acids Res.* 14:5399. Intercalators can also be attached to the 3' end of oligomers, for example as described by U. Asseline et al. (1989), *Tet. Lett.*, 30:2521.

In addition, other substituents can be bound to the 3' end of oligomers by alternate methods. For example, disulfides have been used to attach various groups to the 3' terminus, as described by R. Zuckerman *et al.*, (1987) *Nucleic Acids Res.* 15:5305. It is known that nucleic acids which are substituted at the 3' end show increased stability and increased resistance to degradation by exonucleases (G. Lancelot *et al.* (1985) *Biochemistry* 24:2521; U. Asseline *et al.* (1984), *Proc. Natl. Acad. Sci. USA,* 81:3297).

Nucleic acids also can be synthesized containing a variety of pseudonucleotides or pseudonucleosides which can confer desirable properties such as nuclease resistance. The

pseudonucleotides or pseudonucleosides can also be used as a means to conjugate other molecules to the nucleic acid, such as interchelators. *See* International Patent Application, No. WO 91/13080, Lin, *et al.* entitled "Pseudonucleosides and Pseudonucleotides and their Polymers".

The methods of this invention contemplate a variety of means for delivering the inhibitory nucleic acid to the cell including, for example, direct uptake of the molecule by the cell from solution, facilitated uptake through liposome vectors and intracellular expression from an expression cassette.

One can provide a cell with an inhibitory nucleic acid by contacting the cell with a soluble inhibitory nucleic acid, for example, in the culture medium *in vitro* or in the circulatory system, interstitial fluid or tissue mass *in vivo*. Soluble inhibitory nucleic acids present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species.

In another embodiment, this invention provides expression vectors having an expression control sequence operatively linked to a nucleic acid sequence that encodes an inhibitory nucleic acid that inhibits expression of MIF. An expression control sequence is operatively linked to a nucleic acid sequence when it directs the transcription and translation of that sequence in an appropriate host cell. This includes provision of appropriate start and stop codons.

The choice of expression vectors useful in this invention depends on their intended use. Expression vectors must, of course, contain expression and replication signals compatible with the host cell. Expression vectors useful in this invention include viral vectors, such as retroviruses, adenoviruses and adeno-associated viruses; plasmid vectors; cosmids; liposomes and the like. Viral and plasmid vectors are preferred for transfecting mammalian cells.

Appropriate expression control sequences for mammalian cells include, for example, the SV40 promoter, the RSV (Rous sarcoma virus) promoter and the CMV (cytomegalovirus) promoter.

The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. Sambrook et al., (1989) *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) provide many useful protocols.

Methods of transfecting genes into mammalian cells and obtaining their expression also are well known to the art. See, e.g., *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel, ed.) (1990) or M. Krieger, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, NY, (1990).

This invention also provides methods for inhibiting the activity of MIF in a cell by inhibiting the binding of MIF to Rb protein. One can inhibit the binding of MIF to Rb protein by using compounds that mimic the binding surface of Rb and thereby bind to MIF. These compounds can be, for example, peptides or other organic molecules. The compounds can also be antibodies or engineered binding fragments of antibodies, such as Fab fragments. Small molecules can spontaneously enter cells, while larger molecules may require vectors, such as liposomes, to gain entry.

One can identify such compounds by testing for the ability of the drug to inhibit binding of MIF to Rb protein *in vitro*. Such a test can involve, for example, incubating a sample mixture of the compound to be tested and either MIF or Rb protein, adding Rb protein or MIF, respectively, to the sample mixture, and determining whether the binding of MIF to Rb protein has been inhibited compared to a control sample. Peptides can be identified by screening a peptide library.

In one aspect, methods of inhibiting the activity of MIF are useful in therapeutic treatments for inhibiting the growth of malignant cells in an individual. These treatments involve administering to the individual an effective amount of

an agent that inhibits the mitogenic activity of MIF in the cells.

Agents useful in these methods include, for example, an inhibitory nucleic acid of this invention or a compound that inhibits binding of MIF to Rb protein. In particular, this invention contemplates the use of expression vectors, preferably retroviral vectors, for delivery of the inhibitory nucleic acid molecules of this invention. Methods for delivering nucleic acids as part of genetic therapy are known in the art, as described above. In one embodiment of the invention, one can administer the agent by direct injection into the tumor. One can administer compounds that inhibit the mitogenic activity of MIF in the form of pharmaceutical compositions including the compound and a pharmaceutically acceptable carrier.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous injection. Suitable formulations for injection are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of pharmaceutical compositions comprising agents of the present invention and pharmaceutically effective carriers can be prepared. The pharmaceutical compositions are suitable in a variety of drug delivery systems. For a brief review of present methods of drug delivery, *See*, Langer, (1990) *Science* 249:1527-1533.

The agents of the present invention can be prepared as formulations in pharmaceutically acceptable media, for example, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers.

Systemic administration can also be by transmucosal or transdermal means, or the agents can be administered

orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrations are generally known in the art, and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays, for example, or using suppositories. For oral administration, the agents are formulated into conventional oral administration such as tablets, capsules and tonics.

In preparing pharmaceutical compositions of the present invention, it can be desirable to modify the complexes of the present invention to alter their pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, *See*, *Remington's Pharmaceutical Sciences*, *supra*, Chapters 37-39. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art (*See*, *e.g.*, Langer, *supra*). Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers.

For example, inhibitory nucleic acids can be incorporated into liposomes in order to enhance their pharmacokinetics and biodistribution characteristics. Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano, (1975) *Biochem. Biophys. Res. Commun.* 63:651) and thus having shorter half-lives in the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. For instance, liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream are particularly preferred.

A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al.*, (1980) *Ann. Rev. Biophys. Bioeng.* 9:467, U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028, all of which are incorporated herein

by reference. Typically, the liposomes are prepared with about 5-15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidylinositol. Added negatively charged phospholipids, such as phosphatidylglycerol, also serve to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50 mole percent, and 5-15 mole percent of monosialylganglioside, can provide increased circulation of the liposome preparation in the bloodstream, as generally described in U.S. Pat. No. 4,837,028, incorporated herein by reference. Additionally, the liposome suspension can include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as αtocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

Following liposome preparation, the liposomes can be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. Several techniques are available for sizing liposome to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323.

Following the above treatment, the liposome suspension is brought to a desired concentration for use in intravenous administration. This can involve re-suspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration and the liposomes can be administered parenterally or locally in a dose which varies according to, e.g., the manner of administration, the drug being delivered, the particular disease being treated, etc.

For pharmaceutical compositions which comprise the agents of the present invention, the dose will vary according to, e.g., the particular agent, the manner of administration,

the particular disease being treated and its severity, the overall health and condition of the patient, and the judgment of the prescribing physician.

The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, and capsules.

The pharmaceutical compositions can be administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a solution of the agent dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, and the like. For instance, phosphate buffered saline (PBS) is particularly suitable for administration of soluble complexes of the present invention. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally

employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient.

For aerosol administration, the complexes are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

For topical administration, the agents are formulated into ointments, creams, salves, gels as is generally known in the art. *See, Remington's Pharmaceutical Sciences, supra.*

In therapeutic applications, compositions are administered to an individual in an amount sufficient inhibit growth of a malignant cell. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

The following Example is intended to illustrate but not limit the invention.

EXAMPLE

MIF is induced by two growth factors whose effects are signalled through tyrosine kinase receptor pathways (A. Lanahan et al. (1992) *Mol. Cell. Biol.* 12:3919; A. Ullrich and J. Schlessinger (1990) *Cell* 61:203). To determine whether this effect is specific to this class of receptor signaling pathway, the effect of PDGF on MIF expression in NIH 3T3 cells was compared to that of TGFβ1, another growth factor which is mitogenic in these cells and whose effect is signalled through a serine/threonine kinase system (J. Massague (1992) *Cell* 69:1067). NIH 3T3 cells were grown serum-free (control) or in the presence of either 10 ng/ml TGFβ1 overnight, or 5 ng/ml PDGF for 6 hours.

For northern blot analysis, total RNA was extracted from growth factor treated or control cells. 10 μg of total RNA was used per lane and the blot was probed with ^{32}P-labelled mouse MIF cDNA (G.J. Wistow et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1272). Blots were normalized by hybridization with GAPDH cDNA (ATCC, Rockville, MD). Densitometry was performed on a Bioscan scanner (USB, Cleveland, OH).

Treatment with either growth factor increased endogenous MIF mRNA 2- to 4.5-fold over starting levels (Fig. 1). This induction of MIF mRNA occurs between 2 and 6 hours after the onset of treatment with PDGF, consistent with a delayed early response as observed previously (A. Lanahan et al. (1992) *Mol. Cell. Biol.* 12:3919).

Next, a recombinant MIF gene promoter construct was used to determine whether the induction of MIF mRNA occurs by increased transcription in growth factor stimulated cells. The human MIF gene promoter (V. Paralkar and G. Wistow (1994) *Genomics* 19:48) was cloned upstream of the CAT (chloramphenicol acetyltransferase) reporter gene and stably integrated into NIH 3T3 cells. The human MIF promoter (-1075

to +40) was ligated into the HindIII site of pSV0ATCAT vector (S. Lok et al. (1989) *Nucleic Acids Res*. 17:3563) and transfected by standard calcium phosphate precipitation (C. Gorman et al. (1983) *Science* 221:551) into NIH 3T3 cells, along with the expression plasmid pSV0Neo, containing the neomycin phosphotransferase gene (G418 resistance) as selectable marker. Cells were then grown in the presence of G418 (750 μl/ml) and stably transfected cell clones were selected and subcultured by ring cloning.

For CAT assay, cells were kept serum-free for 24 hours followed by addition of PDGF (109 ng/ml) or TGFβ1 (15 ng/ml). Cells were harvested 12 hours later to obtain extracts for fluor-diffusion CAT assay (J.R. Neumann et al. (1987) *Biotechniques* 5:444) and for protein determination. CAT activity was measured and expressed as activity/min/μg protein. Activity in the absence of serum was used to normalize the activity obtained during various treatments.

When multiple lines of stably transformed cells were treated with either PDGF or TGFβ1, reporter CAT activity was induced 3 to 5 fold (Fig. 2). Thus, the MIF gene is directly induced by two different growth factor signaling pathways suggesting that MIF may have an important role common to cells undergoing mitogenesis stimulated by various agents.

In order to determine the function of MIF protein in proliferating cells, an antisense strategy was used. Antisense and control oligodeoxynucleotides were directly administered to the cell culture medium, an approach which has previously proved successful for suppression of interleukin-1α (J.A. Maier et al. (1990) *Science* 249:1570). Four oligos were used:

aMIF1: GATGAACATA GGCATGGTGG CGGAGAGACT (SEQ ID NO:3);
sMIF1: AGTCTCTCCG CCACCATGCC TATGTTCATC (SEQ ID NO:4);
1FIMa: TCAGAGAGGC GGTGGTACGG ATACAAGTAG (SEQ ID NO:5);
aMIF2: CTCTTATAAA CCATTTATTT CTCCCGGCTG (SEQ ID NO:6).

Oligonucleotide aMIF1 is an antisense to mouse MIF mRNA (A. Lanahan et al. (1992) *Mol. Cell. Biol*. 12:3919; G.J. Wistow et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1272) and centered on the translation start site. Oligonucleotide sMIF1 is the

complement of aMIF1 as a sense control. Oligonucleotide 1FIMa is the reverse of aMIF1 as a control for composition. Oligonucleotide aMIF2 is a second antisense oligo with a similar G+C/A+T ratio to aMIF1, targeted to the polyadenylation signal of mouse MIF. NIH 3T3 cells were grown in the presence of these oligos and treated with growth factors as before (Fig. 3A).

Cell proliferation was assayed using ^{3}H-thymidine incorporation. NIH 3T3 cells in 0.2% serum at a density of $1x10^5$ per ml were seeded in 24 well tissue culture plates at 0.5 ml per well and treated with growth factors and oligos as indicated. After 22 hours, ^{3}H-thymidine was added to the cells and incorporation into DNA was measured for two hours (D. Danielpour et al. (1989) *J. Cell. Physiol.* 138:79). For recovery of cells from aMIF1 treatment, aMIF1-containing medium was replaced by medium containing 10% serum after 22 hours. After a further 22 hours, cells were labelled with ^{3}H-thymidine as before. In the presence of aMIF1, DNA synthesis in response to both PDGF and TGFβ1 was almost completely inhibited while cells grown in the presence of sMIF1, 1FIMa and aMIF2 showed normal levels of incorporation. Cells treated with aMIF1 were not dead, as judged by microscopic inspection, and rapidly regained the ability to respond to mitogenic stimuli when the antisense oligo was removed by washing with fresh serum-containing medium (Fig. 3A).

Western blot analysis of extracts of cells treated with PDGF and TGFβ1 in the presence of aMIF1 showed that MIF protein levels were greatly reduced by antisense treatment (Fig. 3B). This is consistent with the idea that the effect of antisense treatment is exerted through direct suppression of MIF mRNA translation.

The levels of cyclins in antisense treated cells were then examined to localize the effect of MIF suppression in the cell cycle. Cyclins are markers for different parts of the cell cycle. Thus, cyclin-E is a marker for late G1 and is a rate-limiting factor for the G1/S transition, cyclin-A is a marker for S phase and G2 and cyclin-B is a marker for cells

in G2 (M. Ohtsubo and J.M. Roberts (1993) *Science* 259:1908; C.J. Sherr (1993) *Cell* 73:1059). Cells were grown with or without aMIF1 as in Fig. 3. Cell extracts were examined by western blotting with rabbit anti-human cyclin A, B and E antibodies (UBI, Lake Placid, NY) as described above. In PDGF-treated NIH 3T3 cells in the presence of aMIF1 cyclin-B and cyclin-A were greatly reduced while cyclin-E was still detectable (Fig. 4A). This is consistent with cell cycle arrest in G1 and suggests that MIF is required for entry into S phase.

These results were confirmed by flow cytometry of cells grown in the presence and absence of aMIF1 (Fig. 4B). Compared to cells grown in the presence of PDGF, aMIF1 treated cells "piled up" in G1 and there was a marked loss of cells in S phase.

To corroborate the results of antisense suppression the role of MIF in the cell cycle was further investigated by constitutive expression of the recombinant protein. The coding sequence of mouse MIF cDNA was cloned into pMAMNeo (Clontech, Palo Alto, CA) and transfected into NIH 3T3 cells. Stable integrants were selected by G418-resistance. Cells were analyzed by flow cytometry as above. MIF-constitutive NIH 3T3 cells had significantly higher rates of proliferation (Fig. 5) than controls and contained diminished populations in G1 relative to S and G2/M (Fig. 5). This shows that G1 is shortened and that MIF-constitutive cells progress rapidly past cell cycle checkpoints into S phase and begin to accumulate in G2/M. Cells were then deprived of serum. Although control cells ceased proliferation and accumulated in G1, cells constitutively expressing MIF continued to traverse the cell cycle (Fig. 5). This is very similar to the results obtained by overexpressing cyclin-E in mammalian fibroblasts (M. Ohtsubo and J.M. Roberts (1993) *Science* 259:1908). Thus, by itself, MIF mimics the mitogenic effects of serum, consistent with it being a common target for induction by a wide variety of mitogenic agents.

Recently, several small proteins have been identified which inhibit cell cycle progression through

interaction with cyclins (J. Pines (1994) *Trends Biochem. Sci.* 19:143). The activity of MIF is consistent with it being an example of a new class of small proteins which act as positive regulatory subunits of important cell cycle control systems. Since MIF acts late in G1, candidate targets for such regulatory activity are cyclin-E and its partner CDK2 which are essential for G1/S transition and Rb, the product of the retinoblastoma gene, which is an important inhibitor of G1/S transition (R.E. Hollingsworth, Jr., et al (1993) *Curr. Opin. Genet. Dev.* 3:55). The ability of MIF to interact with these potential targets was examined by Western blot analysis of proteins co-precipitated from extracts of serum-treated NIH 3T3 cells by antisera to cyclin-E, CDK2 and Rb. For immunoprecipitation, NIH 3T3 cells grown in the presence of 10% serum were lysed as an incubated in binding buffer (25 mM Tris pH 7.4, 50 mM NaCl, 0.5% Sodium deoxycholate, 0.2% NP-40, 0.2% SDS, 1 mM PMSF, 50 µg/ml aproteinin, 50 µM leupeptin) with either agarose conjugated anti-Rb (Santa Cruz Biotech), or CDK2 (UBI) overnight, or with anti-cyclin E (UBI) for 1 hour followed by addition of protein A agarose and overnight incubation. Agarose beads were then washed three times in binding buffer and boiled in the presence of SDS PAGE sample buffer to release bound proteins, followed by SDS PAGE and Western blot analysis using ECL detection as above. Some rabbit immunoglobulin-derived material was released from the beads and subsequently detected in all lanes by horse-radish peroxidase linked to goat anti-rabbit immunoglobulin (Vector Labs).

No MIF was detected in immunoprecipitates of cyclin-E or CDK2; however, a band of the size expected for MIF was detected by the MIF antiserum in immunoprecipitates of Rb (Fig. 6A). The same result was obtained with two different antisera to Rb. Under non-reducing conditions, the MIF-reactive co-precipitated band increased in size to about 30kDa (Fig. 6B), in agreement with studies suggesting that MIF is a homodimer in solution (J. Nishihira et al. (1993) *Biochem. Mol. Biol. Int.* 31:841). These results are consistent with

MIF exerting its effect at G1/S through an interaction with an Rb-containing complex.

The effect of antisense oligonucleotides on the growth of cancer cells also was tested. The proliferation of cultured cells transformed with *ras*, *raf*, or *sis* oncogenes was essentially halted when the cells were exposed to aMIF1.

All references referred to herein are incorporated by reference in their entirety.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications can be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method useful for inhibiting the growth of a cell comprising the step of inhibiting the mitogenic activity of macrophage migration inhibitory factor ("MIF") in the cell.

2. The method of claim 1 wherein the step of inhibiting comprises providing the cell with an inhibitory nucleic acid that inhibits expression of MIF.

3. The method of claim 2 wherein the inhibitory nucleic acid is directed against a target DNA sequence in the sense strand of the MIF gene.

4. The method of claim 3 wherein the target DNA sequence spans the site of initiation of transcription.

5. The method of claim 2 wherein the inhibitory nucleic acid is directed against a sequence comprising the boundary between an intron and an exon of MIF pre-mRNA.

6. The method of claim 2 wherein the inhibitory nucleic acid is directed against a target sequence in MIF mRNA.

7. The method of claim 6 wherein the target sequence comprises the translational start site of MIF mRNA.

8. The method of claim 7 wherein the inhibitory nucleic acid has the sequence GATGAACATA GGCATGGTGG CGGAGAGACT [SEQ ID NO:3].

9. The method of claim 2 wherein the inhibitory nucleic acid comprises a sequence from the sense strand of the MIF gene or from MIF mRNA.

10. The method of claim 2 wherein the inhibitory nucleic acid induces chemical inactivation of the MIF gene or MIF mRNA.

11. The method of claim 2 wherein the inhibitory nucleic acid cleaves MIF mRNA.

12. The method of claim 2 wherein the step of providing the cell with an inhibitory nucleic acid comprises contacting the cell with the inhibitory nucleic acid.

13. The method of claim 2 wherein the step of providing the cell with an inhibitory nucleic acid comprises transfecting the cell with an expression vector comprising an expression control sequence operatively linked to nucleic acid sequence encoding the inhibitory nucleic acid.

14. The method of claim 1 wherein the step of inhibiting comprises inhibiting the binding of MIF to retinoblastoma ("Rb") protein.

15. The method of claim 1 wherein the cell is a culture cell *in vitro*.

16. The method of claim 15 wherein cell growth is stimulated by the activity of a growth factor.

17. The method of claim 15 wherein cell growth is stimulated by the activity of an oncogene.

18. An isolated inhibitory nucleic acid that inhibits expression of macrophage migration inhibitory factor ("MIF") that is directed against a target sequence selected from the group consisting of a target DNA sequence in the sense strand of the MIF gene, a target sequence comprising the boundary between an intron and an exon of MIF pre-mRNA, and a target sequence in MIF mRNA.

19. The inhibitory nucleic acid of claim 18 wherein the target sequence comprises the translational start site of MIF mRNA.

20. The inhibitory nucleic acid of claim 19 wherein the inhibitory nucleic acid has the sequence GATGAACATA GGCATGGTGG CGGAGAGACT [SEQ ID NO:3].

21. The inhibitory nucleic acid of claim 19 that cleaves MIF mRNA.

22. An expression vector comprising an expression control sequence operatively linked to a nucleic acid sequence that encodes an inhibitory nucleic acid that inhibits expression of macrophage migration inhibitory factor ("MIF") and that is directed against a target sequence selected from the group consisting of a target DNA sequence in the sense strand of the MIF gene, a target sequence comprising the boundary between an intron and an exon of MIF pre-mRNA, and a target sequence in MIF mRNA.

23. A method useful for inhibiting the growth of a malignant cell in an individual comprising administering to the individual a therapeutically effective amount of an agent that inhibits the mitogenic activity of macrophage migration inhibitory factor ("MIF") in the malignant cell.

24. The method of claim 23 wherein the malignant cell expresses the *c-ras*, *c-raf* or *c-sis* oncogene.

25. The method of claim 23 wherein the agent is an inhibitory nucleic acid.

26. The method of claim 23 wherein the agent inhibits the binding of MIF to retinoblastoma protein.

27. The method of claim 23 wherein the step of administering the agent comprises injecting the agent into a tumor mass.

28. A pharmaceutical composition comprising an agent that inhibits the mitogenic activity of macrophage migration inhibitory factor ("MIF") in a cell and a pharmaceutically acceptable carrier.

29. The pharmaceutical composition of claim 28 wherein the agent is an inhibitory nucleic acid.

30. The pharmaceutical composition of claim 28 wherein the agent is a compound that inhibits the binding of MIF to retinoblastoma protein.

31. The pharmaceutical composition of claim 28 further comprising a liposome.

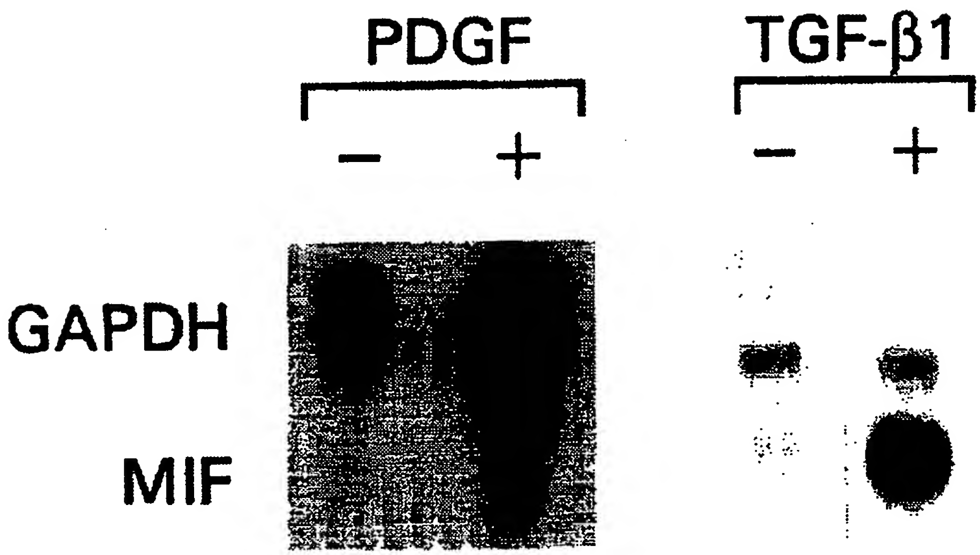

FIG. 1.

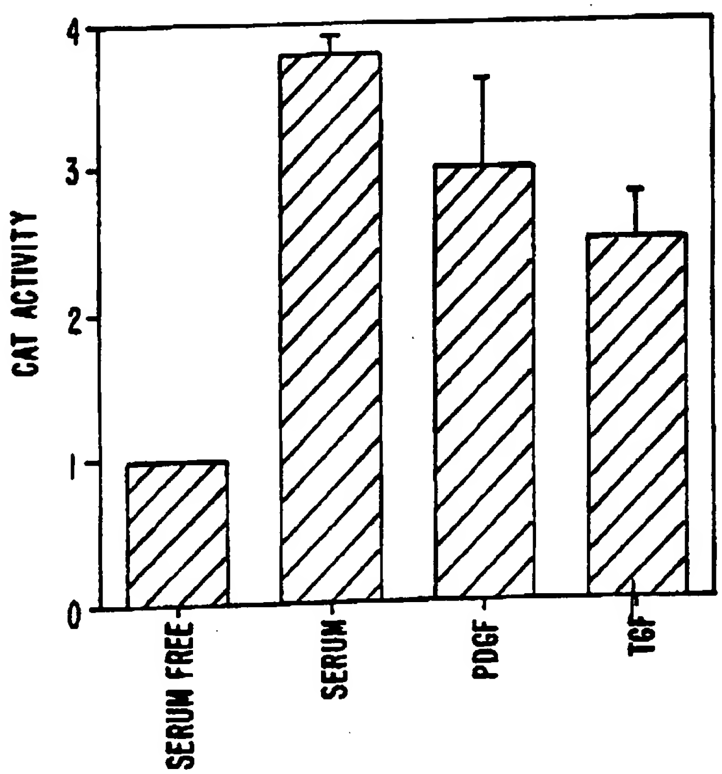
CAT ACTIVITY
0
1
2
3
4
SERUM FREE
SERUM
PDGF
TGF

FIG. 2.

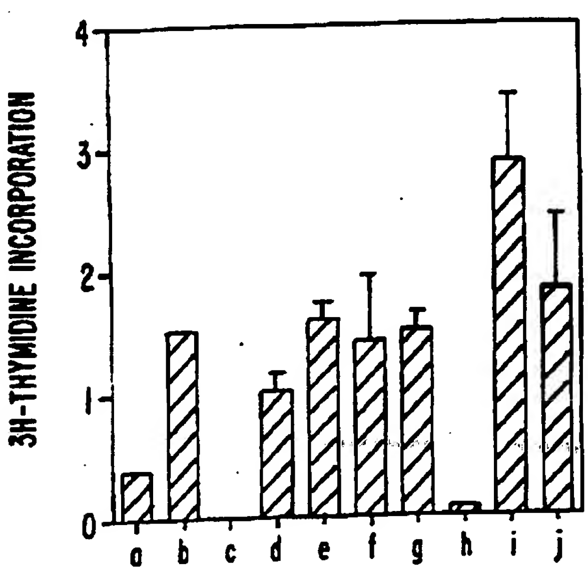
3H-THYMIDINE INCORPORATION
0
1
2
3
4
a
b
c
d
e
f
g
h
i
j

FIG. 3A.

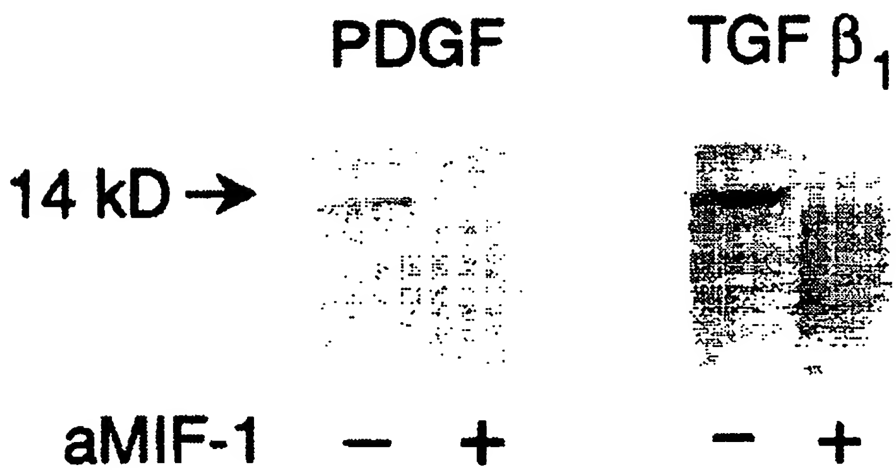

FIG. 3B.

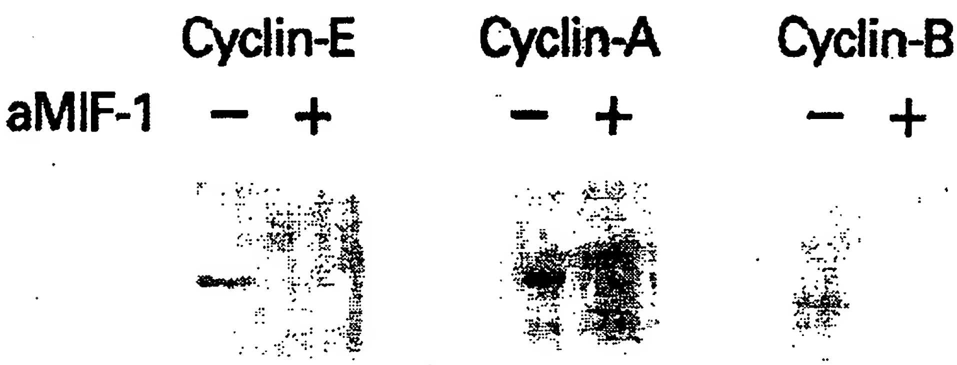

FIG. 4A.

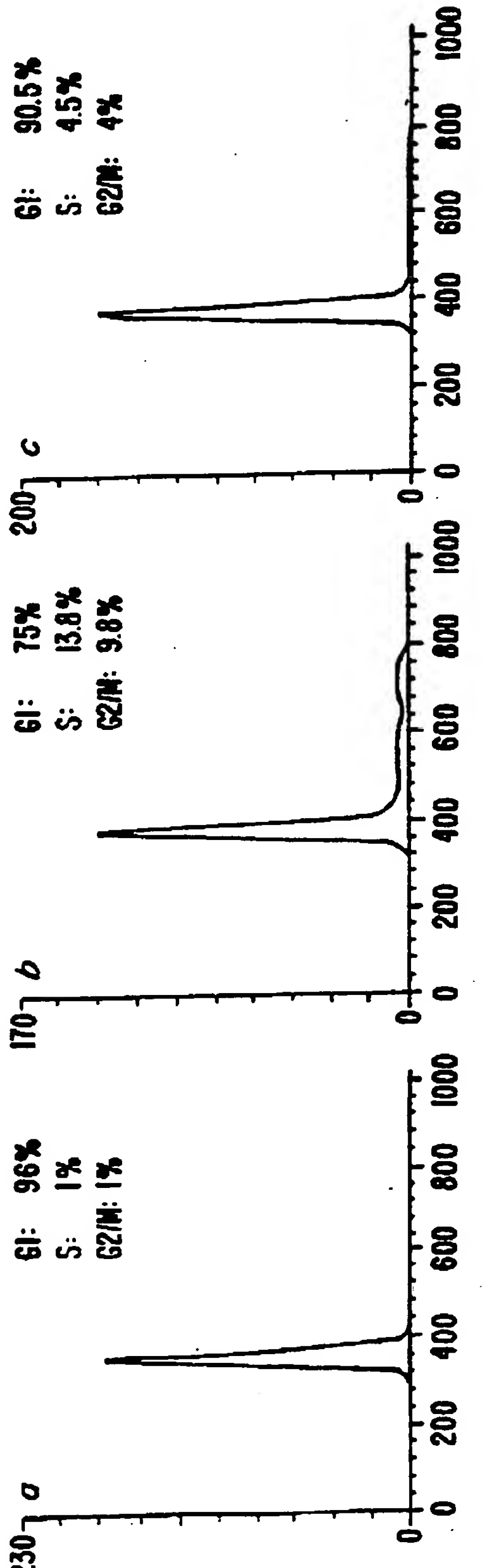
a
230
0
0
200
400
600
800
1000
G1: 96%
S: 1%
G2/M: 1%
b
170
0
0
200
400
600
800
1000
G1: 75%
S: 13.8%
G2/M: 9.8%
c
200
0
0
200
400
600
800
1000
G1: 90.5%
S: 4.5%
G2/M: 4%

FIG. 4B.

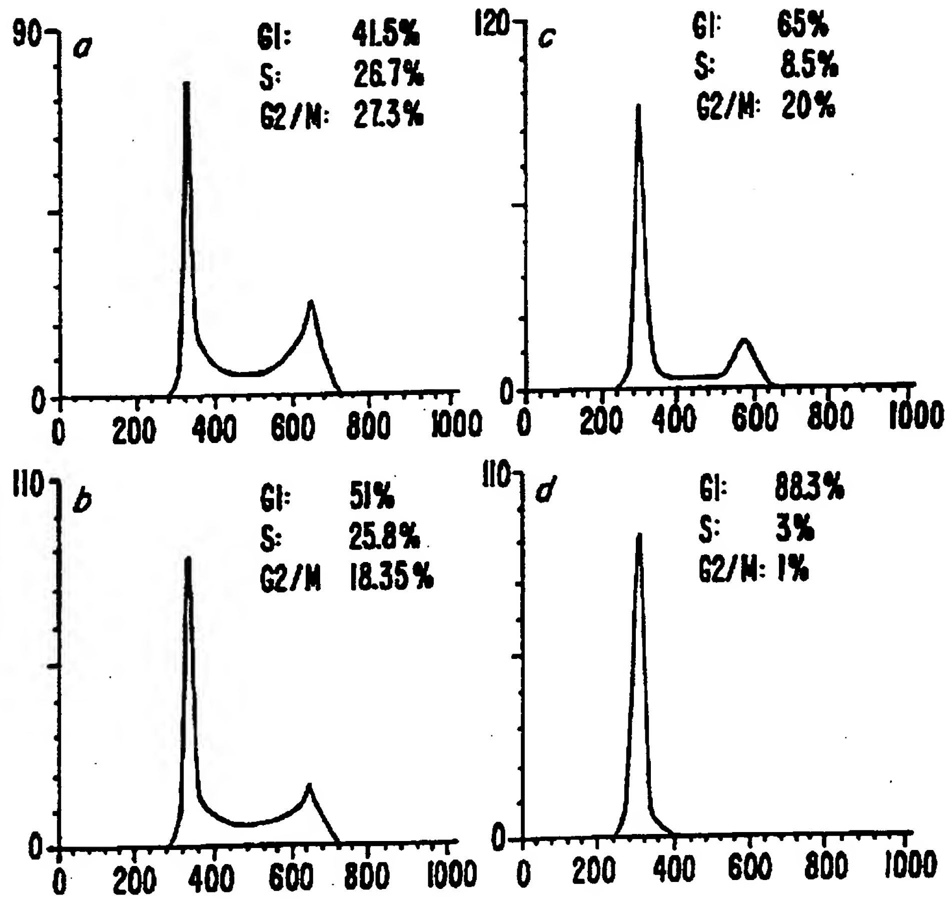
90
a
GI: 41.5%
S: 26.7%
G2/M: 27.3%
0
0 200 400 600 800 1000
120
c
GI: 65%
S: 8.5%
G2/M: 20%
0
0 200 400 600 800 1000
110
b
GI: 51%
S: 25.8%
G2/M 18.35%
0
0 200 400 600 800 1000
110
d
GI: 88.3%
S: 3%
G2/M: 1%
0
0 200 400 600 800 1000

FIG. 5.

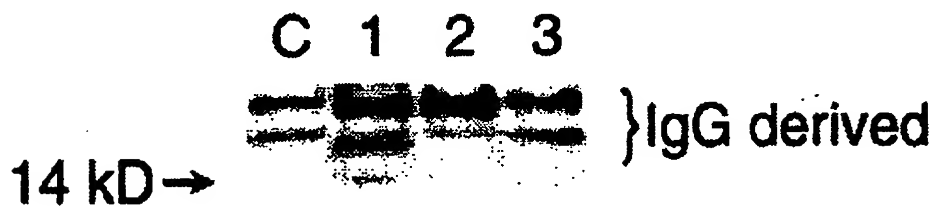

FIG. 6A.

C 1

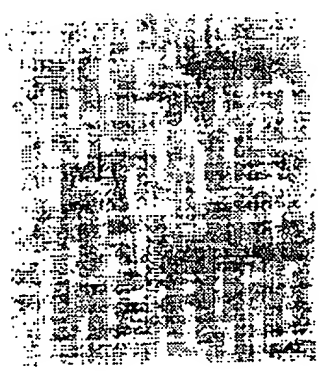

FIG. 6B.